CUTANEOUS AND TRACHEAL RESPIRATION IN THE PHORMIA LARVA

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The larvae of most calypterate flies, of which *Phormia regina* Meig. is an example, have a relatively simple amphipneustic tracheal system consisting basically of a pair of main longitudinal trunks opening to the exterior through one pair of anterior and one pair of posterior spiracles (Fig. 1A, 1B). The anterior and posterior spiracles are strikingly different in structure, but no measurements of their relative importance in the over-all gas exchange of the larva have been reported previously. Similarly, except for the work of Fraenkel and Herford (1938) on *Calliphora,* there are practically no quantitative data on the relative magnitudes of oxygen uptake through the skin and by way of the tracheal system. The *Phormia* larva has proved to be excellent material on which to investigate these problems, particularly because it has remarkable tolerance to radical experimentation: for example, it will live for many hours with either or both ends of the tracheal system closed off by ligatures. Some of the results obtained have been published in abstract (Buck and Keister, 1950a, 1950b; 1953).

MATERIAL AND METHODS

Third instars of *Phormia regina* were collected at about the time of emptying of the digestive tract preparatory to pupation (about 5 days after egg laying, at 25° C.). Culture methods, techniques for holding and ligating the larvae and procedures for stimulating respiration with DDT have been described previously (Buck, Keister and Posner, 1952). In all cases "ligated" refers to larvae with ligatures cutting off one or both pairs of spiracles, whereas "deganglionated" refers to larvae with a ligature just posterior to the brain (which also, of course, closes the tracheae at that point). "Hypoxia" is used to mean a condition in which some aerobic respiration is occurring, but in which oxygen uptake is limited by pO_2.

In applying ligatures there is no direct test of whether the tracheae are actually pinched completely shut, and since the logic of the whole investigation depended on this being true, the ligatures were made tighter than was probably necessary. As previously reported, this not infrequently resulted in one or both of the tracheal trunks actually being severed, particularly in ligating off the anterior spiracles. Less trouble was experienced with deganglionations, since the ligature needed to be only tight enough to prevent leakage when the head end was removed. Whenever blood entered a cut trachea the larva was discarded. Usually, however, the trachea was quickly sealed off at the break point by a small melanotic plug or cap formed at the interface between blood and intratracheal gas. Since the trachea remained *in situ*

and since there was no other visible internal damage—*e.g.,* to the gut—such larvae appeared to be physiologically equivalent to larvae in which the tracheae were not cut, and might even be considered to have the advantage of giving visible proof that no gas could enter the tracheal system through the spiracle in question. Since also, in an extensive series of tests, larvae with cut trunks behaved and respired indistinguishably from those with intact ligated trunks—in sharp distinction to the moribund and severely hypoxic larvae with flooded tracheae—such larvae, representing about 25% of the anteriorly ligated, 10% of the posteriorly ligated, and 10% of the deganglionated animals, were used in the respirometry.
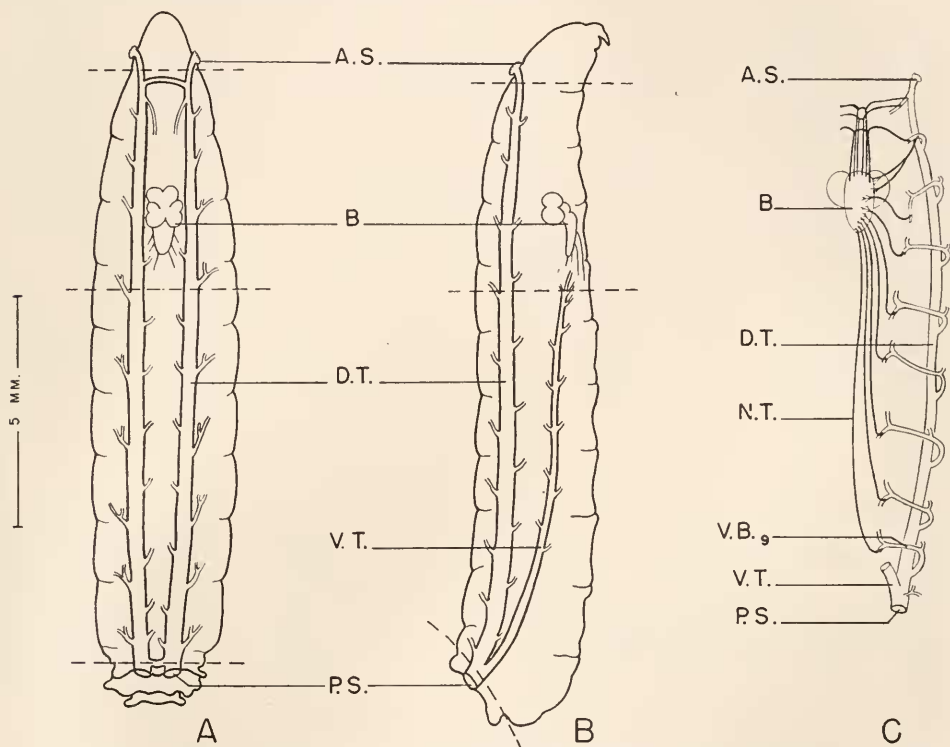

FIGURE 1. A and B, dorsal and lateral diagrammatic views of tracheal system of third stage larva of *Phormia regina* Meig. C, diagram (at slightly less magnification than A and B) of ventral view of neural tracheae (solid black), the dorsal trunk being displaced to the apparent right. A.S. = anterior spiracle; B = brain; D.T. = dorsal trunk; N.T. = neural trachea; P.S. = posterior spiracle; V.B. = ventral branch of dorsal trunk; V.T. = visceral trunk. Dotted lines indicate positions of ligatures.

Improvements made in our technique for administering "light" doses of DDT (Buck, Keister and Posner, 1952) involved using only the minimum clamp pressure necessary to hold the larva (which eliminated leakage due to back pressure), injecting always through the center slit of the left posterior spiracle, and selecting only larvae having DDT solution filling at least the left medial neural trachea (Fig. 1C). With the dose used (approximately 0.6 μL per 60 mg. larva, corresponding to 50 mg./kg. live weight) all larvae were incapable of locomotion at 3½ and at 8

TABLE I

Mean oxygen uptake of *Phormia* larvae in μL/mg. live wt./hr., with standard errors. Figures in brackets are for *Calliphora* at 27° from Fraenkel and Herford, 1938. Inter-group comparisons should be made between same batch numbers (see text). No group of larvae was used for more than one run.

			Unligated			Anterior ligation			Posterior ligation			Double ligation		
		Batch	No. of larvae	Mean uptake	% of control	No. of larvae	Mean uptake	% of control	No. of larvae	Mean uptake	% of control	No. of larvae	Mean uptake	% of control
Unpoisoned	Air	292	18	1.15±.03					17	0.69±.01	60	17	0.09±.003	10
		373	17	0.92±.08								19	0.09±.005	9
		377	20	0.99±.02										
		379	22	0.95±.02										
		383	18	0.67±.02		29	0.95±.04	141						
		384	18	1.04±.07										
		385	17	1.07±.06		16	1.30±.08*	141	16	0.71±.03*	66			
		387	20	0.92±.02										
		388	26	1.07±.03										
		446	54	1.09±.02										
		[4**]		[0.72]		[5**	0.67]	93]				[5**	0.15	21]
	Oxygen	273–4	10	1.24±.05		28	1.10±.07	164	17	1.27±.06	110	16	0.37±.02	39
		292	19	1.11±.04	97	16	1.48±.12*	161	18	1.32±.05*	123	21	0.36±.01	36
		373												
		377												
		383												
		385												
		387										[5**	0.38]	
		388	25	1.10±.02	103									
Poisoned	Air	273–4	20	2.55±.08	206	19	2.50±.05	263	21	1.20±.04	126	18	0.09±.004	10
		373				35	2.38±.05	218	23	1.04±.02	96	20	0.10±.01	10
		377												
		379												
		446	33	2.74±.06	250									
	Oxygen	273–4	18	3.45±.06	278	21	3.37±.08	355	21	2.39±.04	251	18	0.35±.01	38
		373	17	3.21±.05	349	37	3.21±.06	294	22	2.37±.05*	228	22	0.34±.01	34
		377	20	3.58±.12	362				27	2.42±.03	222			
		379	21	3.43±.06	361									
		384	30	3.48±.04	341									
		446	32	3.46±.07	317									

* Another run also made, with almost identical results, on larvae ligated immediately after poisoning and compared with separate contemporaneous controls.

** Indicates number of flasks, each containing about 10 larvae, run by Fraenkel and Herford.

hours after poisoning (tested on wet filter paper) but were often capable of loco-
moting at 24 hours, and were usually successful in forming apparently normal
puparia (although few succeeded in emerging as adult flies).

Oxygen uptake was measured individually in 15-ml. Warburg flasks, using
cylindrical wire screens to keep the control larvae out of the KOH, as described for
adult flies by Buck and Keister (1949). The oxygen uptakes given in the table
are means over a two-hour plateau period, usually between 5½ and 8 hours after
the start of poisoning. Manometers were shaken at about 110 cycles per minute to
aid temperature control.

The schedule of procedures carried out by two workers on the 40-odd larvae used
in an experiment averaged about as follows: hours 0–1, poisoning by intratracheal
injection of 5% DDT in kerosene and checking dosage microscopically; hours
3½–4, weighing and setting up control and unligated poisoned larvae (the latter be-
ing first tested for inability to locomote) in Warburg flasks; hours 3½–4½, ligating,
inspecting and setting up control and poisoned larvae; hours 4½–5, flushing certain
flasks with oxygen (3½–4 L. passed through each flask in 5 min.), leaving others
in air, and equilibration; hours 5–8, recording oxygen uptake at half-hour intervals,
of which the first period was discarded; hours 8–8½, re-inspecting ligated larvae
and testing unligated poisoned larvae for inability to locomote. The present re-
sults include only gas phase respiration: data for respiration in water will be re-
ported elsewhere.

Unless otherwise noted, all experiments were made at 25° C. Statistical signifi-
cance was assessed by t test, using the 2% level.

Results

Oxygen uptakes from gaseous air or oxygen of larvae under various conditions
are given in Table I; those of unpoisoned larvae in the upper half, those of larvae
with DDT-stimulated respiration in the lower half. As in previous work, the vari-
ability in the material made it desirable to give data from a number of different
batches for each category of larva, each with its own control, and to draw all
conclusions from statistical analysis of intra-batch comparisons. To facilitate such
comparisons, mean uptake rates are given both in μL/mg. live wt./hr. and as per-
centages of that of normal controls for the particular batch of larvae. Some groups
seem to be more homogeneous when compared on one basis (e.g., unligated, poisoned
in oxygen) and some on the other (e.g., anterior ligated, unpoisoned).

A. Cutaneous respiration

In gaseous air the oxygen uptake of doubly ligated larvae was approximately
10% of that in unligated controls (Table I). It is thus clear that larvae in which
spiracular pathways of oxygen entry have been eliminated must become severely
hypoxic.

In doubly ligated larvae poisoned intratracheally with DDT, oxygen uptake from
air (10%) was no higher than that of doubly ligated unpoisoned larvae, in striking
contrast to the great increase seen in poisoned unligated or once-ligated larvae in
gaseous air or oxygen (Buck, Keister and Posner, 1952, and below). This, there-
fore, seems to indicate that, regardless of the potential metabolic rate, the maximum

amount of oxygen which can be taken in through the skin from air is of the order of 10% of the normal uptake. It can also be concluded that the presence of DDT in the body does not alter cutaneous permeability to oxygen.

Cutaneous uptake from oxygen was only of the order of four times that in air (39% vs. 10% in both unpoisoned and poisoned larvae). It was not increased by suspending the (doubly-ligated) larvae from the necks of the manometers, so that they swung back and forth inside the flasks as the manometers shook. This indicates that access to environmental oxygen is not the factor preventing the respiration from increasing linearly with pO$_2$.

As pointed out by Fraenkel and Herford (1938), the severe hypoxia in larvae restricted to cutaneous respiration undoubtedly means that the internal pO$_2$ is lower than in normal larvae respiring in air, and the trans-cutaneous oxygen gradient correspondingly steeper. This indicates, therefore, that the observed cutaneous respiration (10%) is higher than in normal larvae. Estimates of the proportion of oxygen taken in through the skin in normal larvae, and of the internal pO$_2$ in larvae respiring only through the skin will be given in the Discussion.

B. *Relative contributions of anterior and posterior spiracles to total oxygen uptake* (*Table* I)

The oxygen uptake from air of unpoisoned larvae with only the posterior spiracles functional (anterior spiracles tied off) was about 40% higher than that of normal unligated larvae, due, presumably, to increased activity induced by the ligature. The uptake from air of larvae with the anterior spiracles, alone, functional was only 60–65% of what is required in normal metabolism. In both types of ligation, of course, some oxygen entered also through the skin, but since the amount was presumably proportional to the environment-tissue gradient it could not have exceeded, and probably was much less than, the amount passing through the skin of the larva with all spiracles blocked (10% of control). This indicates, therefore, that in unpoisoned larvae the posterior spiracles by themselves can pass at least as much air as is normally required, whereas the anterior spiracles by themselves cannot.

The conclusion as to the relative "capacities" of the two pairs of spiracles is supported qualitatively by the facts that (a) normal unligated larvae in osmium tetroxide vapor blacken first and most intensely at the posterior end, and (b) pupating larvae in which the anterior spiracles have been ligated off darken evenly and at normal speed, whereas those with the posterior spiracles, alone, ligated off, darken slowly and unevenly, starting at the front end. Fraenkel (1935) obtained the same effect in *Calliphora* by immersing alternate ends of the larva in oil. Additional more tenuous pieces of circumstantial evidence which are at least compatible with the subordination of the anterior to the posterior spiracles are: (c) in larvae immersed in kerosene, the oil invariably enters the tracheal system through the posterior spiracles; (d) in larvae held under water and pinched at one end or the other in such a way as to close one pair of spiracles, gas can be driven out of the posterior spiracles with relative ease, but out of the anterior spiracles only with considerable difficulty. A necessarily smaller gas volume passed by the anterior spiracles can perhaps also be deduced from the anatomical facts that (e) the diameter of the main tracheal trunk at the posterior spiracles is about 285 μ, whereas at the anterior spiracles it is only 135 μ, and (f) the only tracheal supply to the digestive tract

comes off the main trunks at the extreme posterior end (visceral trunk, Fig. 1B), thus necessitating a long detour for gas supplied via the anterior spiracles.

C. *Effects of DDT and of oxygen on oxygen uptake in unligated and once-ligated larvae* (*Table* I)

After administration of DDT, oxygen uptake from air increased markedly in both anteriorly and posteriorly ligated larvae, and in unligated larvae. This is consistent with the increase in muscular activity characteristic of DDT stimulation (*cf.* Buck, Keister and Posner, 1952), which lowers the internal pO_2 and increases the environment-tissue oxygen gradient.

As set forth in Table I, normal larvae took up oxygen at the same rate from pure oxygen as from air, showing that oxygen is neither limiting nor stimulating to normal respiration in air. In all other groups, both poisoned and unpoisoned, oxygen uptake from oxygen was significantly higher than from air. As applied to the unpoisoned anteriorly ligated larvae this finding is perhaps a little surprising since
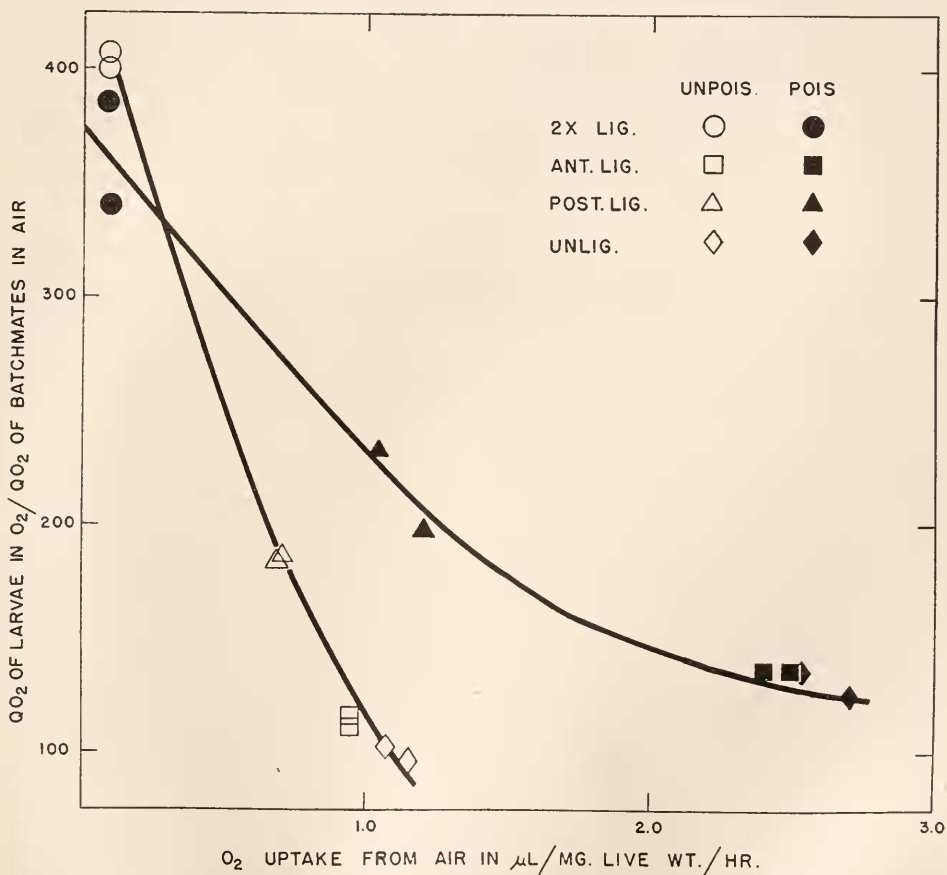

FIGURE 2. Relation between the rate of O_2 uptake from air by larvae with various types of respiratory restrictions (abscissae) and the degree (percentage) by which this is surpassed by the uptake of similar larvae in pure oxygen (ordinates). Data from Table I.

it indicates that even the stimulation due to the ligature makes the larva mildly hypoxic, and that the posterior spiracles alone, though adequate for control respiration, are only just so.

The metabolic stimulation due to DDT is apparently so great that even with four times as much oxygen available neither pair of spiracles by itself is adequate.[1] As a matter of fact, even the unligated poisoned larvae in oxygen might prove to be hypoxic—i.e., capable of a higher rate of oxygen uptake—if tested in pure oxygen at greater than atmospheric pressure.

It is understandable that the absolute respiratory rates of the poisoned larvae, both in air and in oxygen, should be in the order unligated > anteriorly ligated > posteriorly ligated > doubly ligated, since this reflects the order of the "capacities" of the available pathways of oxygen entry. On the other hand, the fact that the *percentage* increases in uptake shown by the various groups in oxygen as compared with air are in the reverse order in both unpoisoned and poisoned larvae is consistent with the expected order of severity of hypoxia. Thus the most hypoxic groups, the doubly ligated larvae, take up four times as much oxygen from pure oxygen as batchmates do from air, whereas the poisoned unligated larvae, which have all their normal respiratory avenues intact, show only about a 30% increase. The non-linearity of the *increase* in respiration with five-fold increase in pO_2 is well shown in Figure 2. Though unexplained, the curious inability of the poisoned singly ligated larvae to take up as much oxygen from oxygen as the unligated poisoned larvae (*i.e.*, to fully eliminate hypoxia) deserves attention.

Discussion

Other things being equal, it might be expected that the oxygen uptakes of the 60-mg. *Phormia* larva and the morphologically similar 80-mg. larva of *Calliphora* would be of the same order of magnitude. As shown in Table I this expectation is borne out fairly well for most of the comparable types of experiment in spite of the considerable differences in experimental techniques. One minor discrepancy is that Fraenkel and Herford (1938) reported uptakes from air slightly below normal in *Calliphora* larvae with the anterior spiracles ligated off, whereas we observed in *Phormia* an uptake about 40% above the control, which we attribute to the greater activity caused by the irritation of the ligature. Another difference is in the apparent proportion which cutaneous respiration forms of the total oxygen uptake from air (21% in *Calliphora*; 10% in *Phormia*), which is especially surprising in view of the fact that in *Calliphora* respiration was measured on samples of 10 larvae simultaneously, where constant mutual contact stimulation might have engendered an artificially high level of control activity. Possibly the difference is due to Fraenkel and Herford's having followed respiration for only an hour, a period which, in *Phormia,* is marked by violent struggling of the doubly ligated larvae, whereas all our calculations represent the means of the uptakes over a two-hour period several hours after the start of the experiment. However, there may well be a real interspecific difference in cutaneous permeability to oxygen, particularly in view of the

[1] In batch 379 the O_2 uptake of unligated poisoned larvae in oxygen was not significantly higher than that of the anteriorly ligated poisoned larvae in oxygen (3.43 vs. 3.37), but the difference between the corresponding groups in batch 446 is so great that a highly significant difference (p = < 0.01) is obtained even if the two batches are lumped. It is evident that the anteriorly ligated larvae in oxygen are able to take in almost enough oxygen to avoid hypoxia.

difference in thickness of the cuticles and in reaction to paraffin-alcohol mixtures (Fraenkel, personal communication).

Fraenkel and Herford attempted to circumvent the effect of voluntary activity by measurements on deganglionated larvae. These respired at a rate which was about half that of normal controls and which was assumed to be the basal rate. They found that the oxygen uptake of deganglionated larvae with the hind spiracles also ligated off was about one quarter of that of deganglionated larvae with functional rear spiracles (thus one-eighth that of normal larvae). As already pointed out, this would actually correspond, in normal (non-hypoxic) controls, to much less than an eighth (12%) of the total uptake. By indirect methods Fraenkel and Herford estimated that the true cutaneous respiration is about 10% of the basal (hence 5% of the normal control). Buck, Keister and Posner (1952) found a drop of 32% in the oxygen uptake of deganglionated *Phormia* larvae at 25° in one batch, and a nonsignificant drop of 7% in another. In check experiments for the present paper, each involving 40–50 larvae, we found a nonsignificant drop of 4% and a nonsignificant increase of 3% at 25° and a significant drop of 16% at 30°. Since Fraenkel and Herford did not check their larvae for leakage of blood into the tracheae, which occurs rather frequently in *Phormia,* it is possible that the presumed basal rate in *Calliphora* is too low because it represents an average of the uptakes of larvae with flooded tracheae (and hence respiring at the very low rate of twice-ligated larvae), and others with rates close to that of unligated controls.

The increased cutaneous respiration in doubly ligated larvae in oxygen (Table I) is the expected response of these severely hypoxic animals to increased environmental pO_2. The unchanged cutaneous respiration after poisoning is therefore especially significant, not only because it shows that DDT has not affected cutaneous permeability to oxygen, but because it indicates that DDT has not significantly affected the oxygen gradient between environment and tissues (assuming that static diffusion is the mechanism of gas transport across the integument). The simplest explanation of the non-effect of DDT is that the internal pO_2 is already zero even in the unpoisoned doubly ligated larva, and oxygen is hence already penetrating the integument at its maximum rate for the oxygen gradient of about 136 mm. Hg, which obtains in the Warburg flask at 25°. This is what Fraenkel and Herford (1938) had postulated for doubly ligated *Calliphora* larvae, on the basis of their work with varied pO_2. As a matter of fact, the equality of oxygen uptake in unpoisoned and poisoned doubly ligated *Phormia* larvae in oxygen (Table I) shows that even with a trans-cutaneous gradient of 737 mm. the tissue pO_2 is essentially zero, indicating *a fortiori* that it must be zero in such larvae respiring air.

By making use of the oxygen uptakes measured at the known gradients, the permeability of the integument to oxygen can be calculated. Thus, using the mean total skin area of 8 doubly ligated 60-mg. larvae (85 mm.²), the permeability for air respiration (0.09 μL/mg. live wt./hr.) is 0.0056 μL/mm.²/min./atm., and the value for oxygen 0.0042. A. G. Richards (personal communication) found good agreement between cutaneous permeability calculated from Warburg measurements on two doubly ligated larvae of *Sarcophaga bullata* in O_2 (the absolute values being about the same as in *Phormia*) and that calculated from oxygen electrode measurements on the isolated cuticles of the same larvae, using O_2-saturated water on one side and N_2-saturated water on the other. This supports the suggestion that the

tissue pO_2 in doubly ligated larvae quickly falls to zero and that the recorded rate for cuticular penetration is indeed maximal.

Assuming that neither DDT nor oxygen causes a physical change in the pathways of oxygen entry, it seems reasonable to postulate that if oxygen uptake via the three possible routes (skin, anterior spiracles, posterior spiracles) could be measured in larvae in which the internal pO_2 was the same, the ratio skin : ant. : post. would be the same as in the normal larva even though the internal pO_2 in the experimental animals was different from the normal. In such circumstances one would expect the following arithmetical relation to hold : total uptake (*i.e.,* unligated larva) = (cutaneous uptake) + (uptake of anteriorly ligated larva minus cutaneous uptake) + (uptake of posteriorly ligated larva minus cutaneous uptake). In the present series of experiments it seems clear that a strict quantitative separation of the avenues of oxygen entry is not possible, because the ligating which is necessary to separate the three pathways causes different degrees of hypoxia and hence modifies the inwardly directed oxygen gradients. This is indicated, for example, by the relatively poor fit obtained in substituting, in the above equation, the oxygen uptakes for the most severely hypoxic groups (poisoned, in air) of batch 446, using any value up to 0.1 for the small cutaneous correction factors (x_1, x_2, x_3) :

$$2.74 = x_1 + (2.36 - x_2) + (1.04 - x_3).$$

A rigorous budget could presumably be made out either (1) by subjecting the four classes of larvae to reduced pO_2 and comparing their oxygen uptakes at the highest respective pO_2s at which DDT had no stimulating effect (*i.e.,* reducing all groups to zero internal pO_2) or (2) by comparing the uptake from air of each group at the highest temperature at which flushing with pure oxygen had no effect (*i.e.,* lowering, in most cases, the temperature until the metabolic requirements could be met by the amount of oxygen which could enter through the restricted avenues). Experiments along both the lines suggested above are under way, but for present purposes useful approximations can be derived from the data already available (Table I). For example, if we average the uptakes of the two batches in each of the ligated poisoned groups in air we get, neglecting the corrections for cutaneous uptake, a cutaneous : anterior : posterior ratio of 0.095 : 1.12 : 2.44, or approximately 1 : 12 : 26. As already discussed, we know that the cutaneous contribution must normally be much less than in doubly ligated larvae, but this at least shows us at once that it cannot be *more* than 1/39 (2.5%) of the total. Furthermore, although the indicated uptake through the anterior spiracles alone should be proportionately too high, both because the posteriorly ligated animal is more hypoxic than the anteriorly ligated and because of the irritating effect of the ligature, an A : P ratio of 12 : 26 or 1 : 2 is probably roughly correct. This is indicated by the facts (1) that the A : P ratio is approximately the same in both unpoisoned and poisoned larvae in air ; (2) that the percentage increase in respiration in oxygen as compared with air in the poisoned anteriorly ligated larvae (130%) is close enough to that in the poisoned posteriorly ligated larvae (210%) to indicate that their hypoxias are at least of the same order of magnitude ; and (3) because the observed uptake through the anterior spiracles is undoubtedly lowered, as compared with normal, because the ligature cuts off the visceral tracheal trunks from the main longitudinal trunks at the same time it closes the posterior spiracles (Fig. 1). Furthermore, in experiments in which oxygen intakes by the two ends of the body were measured separately in

normal control larvae stuck through a rubber membrane, an A : P ratio of approximately 2 : 3 was observed (Buck and Keister, unpublished data).

The present evidence of the subordinate physiological position of the anterior spiracles agrees also with opinions of Strasburger (1935) on *Drosophila* and Levenbook (1951) on *Gastrophilus*.

None of the evidence on the relative "capacities" of the two pairs of spiracles bears on the question of whether gas transport is via diffusion or ventilation. This problem will be dealt with elsewhere.

SUMMARY

1. In the *Phormia regina* larva oxygen uptake from air at 25° of larvae with both pairs of spiracles ligated off is about 0.09 μL/mg. live wt./hr. (10% of normal respiration) and increases four-fold in oxygen. This is the maximum cutaneous uptake possible from air.

2. The posterior pair of spiracles by itself can admit enough oxygen from air to supply normal needs.

3. On the basis of reciprocal ligation experiments, the anterior spiracles have about half the "capacity" of the posterior spiracles for admitting oxygen into the tracheal system, and can, by themselves, admit about 60% of normal needs.

4. The internal pO_2 in larvae with both pairs of spiracles ligated is zero, even in an atmosphere of pure oxygen.

5. In normal (non-hypoxic) larvae, cutaneous respiration accounts for less than 2.5% of the total oxygen uptake.

6. The permeability of the integument to oxygen is of the order of 0.005 μL/mm.^{2}/min./atm.

7. DDT does not affect cutaneous permeability to oxygen.

8. DDT-poisoned larvae cannot obtain enough oxygen from air even with all spiracles and skin functional, the hypoxia increasing nonlinearly in the order unligated < anteriorly ligated < posteriorly ligated < doubly ligated. Even in pure oxygen the larvae are hypoxic if either pair of spiracles is cut off.

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